ENSILING PROCESS II. ECOLOGICAL ASPECTS OF YEAST ACTIONS IN THE SILAGED PROCESS

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By

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(Received January 15, 1955)

Introduction

In a previous report (1) the author published his results concerning the putrefied process in the ensiled pile, from which the yeast was always isolated. At that time it was assumed that, when the sugars once accumulated in the pile that has been exhaustively consumed by the yeast living therein and the accumulated acid is utilized by the yeast as an energy source if the aerobic conditions are active, so that the acidic environment converts into an alternative side, which in turn may favor the growth of the aerobic putrefying bacteria. However, if the anaerobic environment is well supplied, the acid is not exhausted and is sufficient to prevent the bacterial growth. Thus the yeast may display a significant rôle in the fields of whether or not putrefaction occurres.

The yeast isolated from the ensiled pile by using the culture medium can oxidize glucose and acetic or lactic acids as well, which usually are the chief acids formed during the ensiling process. This fact may partially confirm the above assumption, but there remains a gap between the actual phenomena and the laboratory facts. This gap may be covered by observations on the actual yeast action during the ensiling process. Such an ecological observation is done by the author with the technique which has been recently adapted to the research on the Sake-yeast in our laboratory (2) (3), and the results are presented in this report.

Materials and Methods

Isolation of the so called "silage-yeast".

Almost all of the samples so far tested contained both bacteria and yeast. The yeast therein present was collected by differential centrifugation, as follows. 100 g of a sample was crushed through a mincer, then shaken for 30

minutes after the addition of five volumes of distilled water, and then differentiated into three portions by centrifugation. Almost all of the plant tissues contained in the samples were deposited after centrifugation for one minute at 500 r.p.m. From this supernatant the yeast fraction was obtained by centrifugation for five minutes at 1000 r.p.m., and the bacterial fraction by that for 30 minutes at 3000 r.p.m. The obtained yeast fraction was somewhat contaminated by either the plant tissues or bacteria, and when washed thrice with distilled water by centrifugation at the same r.p.m. as adopted in yeast fractionation to remove as much contaminated bacteria as possible, it contained a volume of the plant tissues so scanty, that the measurement of the yeast activity was not hindered and therefore it was neglected. Such a yeast fraction which was obtained without any culture medium could be treated like the purely isolated yeast, and accordingly it was designated "silage-yeast" to distinguish it from the ordinary laboratory yeast.

Counting of the yeast cells and measurement of their metabolic activities.

The yeast fraction above obtained was re-suspended in 10 ml of sterile water, and a part of this yeast suspension was employed for cell counting by the plate method (viable counting). The remaining part was subjected to the metabolic test, which was carried out manometrically at 37° in a Warburg vessel. Components of the vessel were as follows unless otherewise stated: yeast suspension 1 ml, M/5 phosphate buffer (pH 3.5) 2.0 ml, M/35 substrate. Respiratory (Xo_2) as well as aerobic and anaerobic fermentative activities ($X^{air}_{CO_2}$ and $X^{N_2}_{CO_2}$) of the yeast, respectively, were examined for 60 minutes (or longer if necessary), where glucose, sodium lactate, and also free lactic and acetic acids were used as a substrate, resperctively. If necessary, cell activity was calculated by division of the above obtained activities of the yeast with its cell number. Then the usual expression of the yeast activities, such as Xo_2, $X^{air}_{CO_2}$ and $X^{N_2}_{CO_2}$ refers to the total metabolic activity of the yeast cells present in 1 g of the silage sample.

Results

Previously the author pointed out that the ensiling process is distinguishable into several aging phases according to the changes in its chemical constituents and the microbes therein present. Cell number as well as metabolic activites of the yeast present in each silage sampled varied in corespondence with its aging phases. The silage sample A, which was prepared from green maize of the Farm in 1952, was investigated as to cell number and the oxidative activity of the yeast contained. In the silage sample B was piled green soybean besides green maize which were obtained from the Farm in 1953. The yeast therein present was examined regarding its oxidative and fermentative activities besides its cell number, so that a precise stature of the changes in the yeast metabolic

Table 1. Vaiable numbers of the "Silage-yeast" and their metabolic actvities in the Ensiling Processes

Silage A.

days	4th						8th						15th						25th						100th					
Portion	pH	cells 10^5/g	substrate		I	II	pH	cells 10^5/g	substrate		I	II	pH	cells 10^5/g	substrate		I	II	pH	cells 10^5/g	substrate		I	II	pH	cells 10^5/g	substrate		I	II
Surface	4.6	2.0	Xo_2	glucose lactic Na-lactate acetic	2.4 0 6.6 0	1.2 0 3.3 0	4.5	240	Xo_2	glucose lactic Na-lactate acetic	240 144 110 145	1.0 0.6 0.45 0.6	4.6	7.5	Xo_2	glucose lactic Na-lactate acetic	7.5 2.5 0.45 0	1.0 0.35 0.6 0	7.4	6.0	Xo_2	glucose lactic Na-lactate acetic	0 0 8.5 0	0 0 1.4 0	7.6	1.2	Xo_2	glucose lactic Na-lactate acetic	6.0 2.5 14.0 2.4	5.0 2.1 14.0 2.0
			$X^{air}_{co_2}$ $X^{N_2}_{co_2}$	(glucose)	-- 13				$X^{air}_{co_2}$ $X^{N_2}_{co_2}$	(glucose)	-- --				$X^{air}_{co_2}$ $X^{N_2}_{co_2}$	(glucose)	12 39				$X^{air}_{co_2}$ $X^{N_2}_{co_2}$	(glucose)	0 0				$X^{air}_{co_2}$ $X^{N_2}_{co_2}$	(glucose)	-- --	
Center	4.1	3.5	Xo_2	glucose lactic Na-lactate acetic	6.3 0 22.0 0	1.8 0 6.3 0	3.6	15	Xo_2	glucose lactic Na-lactate acetic	16.5 0 12.8 0	1.1 0 0.25 0	3.5	5.5	Xo_2	glucose lactic Na-lactate acetic	4.8 5.2 3.8 0	0.95 0.95 0.65 0	3.4	2.6	Xo_2	glucose lactic Na-lactate acetic	11.2 -- -- --	5.6 -- -- --	3.6	2.3	Xo_2	glucose lactic Na-lactic acetic	7.4 -- 2.5 --	3.2 -- 1.1 --
			$X^{air}_{co_2}$ $X^{N_2}_{co_2}$	(glucose)	-- 150				$X^{air}_{co_2}$ $X^{N_2}_{co_2}$	(glucose)	-- --				$X^{air}_{co_2}$ $X^{N_2}_{co_2}$	(glucose)	-- --				$X^{air}_{co_2}$ $X^{N_2}_{co_2}$	(glucose)	4 1				$X^{air}_{co_2}$ $X^{N_2}_{co_2}$	(glucose)	-- --	

Silage B.

days	4th						8th						20th						35th						123th					
Portion	pH	cells 10^5/g	substrate		I	II	pH	cells 10^5/g	substrate		I	II	pH	cells 10^5/g	substrate		I	II	pH	cells 10^5/g	substrate		I	II	pH	cells 10^5/g	substrate		I	II
Surface	4.6	18	Xo_2	glucose lactic Na-lactate acetic	210 270 300 0	11.7 15.0 16.7 0	4.8	350	Xo_2	glucose lactic Na-lactate acetic	144 221 87 0	0.4 0.6 0.3 0	4.5	12.4	Xo_2	glucose lactic Na-lactate acetic	4 5 12 0	0.03 0.04 0.9 0	4.5	3.8	Xo_2	glucose lactic Na-lactate acetic	25 40 43 33	6.6 10.5 11.3 8.9	5.0	7.5	Xo_2	glucose lactic Na-lactate acetic	10 9 14 0	1.3 1.2 1.9 0
			$X^{air}_{co_2}$ $X^{N_2}_{co_2}$	(glucose)	1560 2110				$X^{air}_{co_2}$ $X^{N_2}_{co_2}$	(glucose)	14 142				$X^{air}_{co_2}$ $X^{N_2}_{co_2}$	(160′) (glucose) (60′~120′) lag:60′	18 30				$X^{air}_{co_2}$ $X^{N_2}_{co_2}$	(glucose)	9 1				$X^{air}_{co_2}$ $X^{N_2}_{co_2}$	(glucose)	-- --	
Center	4.2	9.0	Xo_2	glucose lactic Na-lactate tcetic	258 380 235 0	31.7 42.2 26.1 0	3.5	9.0	Xo_2	glucose lactic Na-lactate acetic	35 26 32 0	3.9 2.9 3.6 0	3.5	8.7	Xo_2	glucose lactic Na-lactate acetic	12 6 13 0	1.4 0.7 1.5 0	3.5	1.0	Xo_2	glucose lactic Na-lactate acetic	8 10 33 17	8.0 10.0 33.0 17	3.5	1.0	Xo_2	glucose lactic Na-lactate acetic	32.5 40 -- --	32.5 40 -- --
			$X^{air}_{co_2}$ $X^{N_2}_{co_2}$	(glucose)	1400 1050				$X^{air}_{co_2}$ $X^{N_2}_{co_2}$	(glucose)	65 65				$X^{air}_{co_2}$ $X^{N_2}_{co_2}$	(glucose)	28 61				$X^{air}_{co_2}$ $X^{N_2}_{co_2}$	(glucose) (40′~100′) lag:40′	6 7				$X^{air}_{co_2}$ $X^{N_2}_{co_2}$	(glucose)	-- --	

Silage A was prepared from green maize alone in 1952.

Silage B was prepared from green maize and soybean in 1953.

* Column I indicates the total metabolic activities of the yeast cells present in 1 g of the silage sample.

** Column II indicates the activities per 10^5 yeast cells.

activities during the ensiling process was obtained mainly in the sample B (Table 1). Both surface and center portions of each silage were collected for research.

Phase 1. This phase is characterized by a growing stage of the bacteria contained in the ensiled matters, where pH of the ensiled pack was slightly lowered. This is an initial stage of the ensiling process and extends to the fourth day after packing. The yeast in both portions of the silage either began to grow (silage A), or was increasing its growth rate (silage B), and accordingly the yeast of the former showed metabolic activities lower than those of the latter. In the silage A an anaerobic fermentability of its surface yeast (the silage-yeast at the surface portion of the silage) was much smaller than that of its inner yeast (the silage-yeast at the center portion of the silage), whereas both silage yeasts indicated approximately identical but faint activity for glucose oxidation. In the silage B, both surface and inner yeasts in this phase were the most active in their respiratory and fermentative abilities throughout the ensiling process. Particularly, both yeasts showed an extremely high anaerobic fermentative activity, which exceeded about five to tenhold more than their respiratory one. This latter oxidative ability of either of both yeasts was inert at the beginning of their respective reactions with glucose, and after about 30 minutes exerted an activation, whereas their anaerobic fermentability entered into the stationarily activated state already from the beginning of each of these reactions, as shown in Figs. 1 and 2. The author previously observed that an aerophilic yeast isolated from the silage (film-forming yeast strain No. 32) shows much lower anaerobic fermentability in comparison with its oxidative activity, which in turn was inert only for a monent (less than 10 minutes) at the beginning of the reaction with glucose. The reverse, however, was the case in an anaerophilic yeast also isolated from the silage (non-film-forming strain No. 39) which indicated considerably high anaerobic fermentability and a much longer time of lag before active oxidation toward glucose. Cells of these laboratory yeasts thus employed were collcctcd from the shake culture in glucose-peptone-medium. When the pressed juice of the ensiled pile of days old, packed with green soybean and maize, was supplied with 2 per cent of the glucose and then used as a culture medium after filtration through Seitz's filter, the glucose-silage juice produced rather a delaying effect upon the growth of both above laboratory yeasts and required an abandant volume of inoculum size to cause their sufficient growth. The aerophilic yeast strain No. 32, as cultured in this medium by standing instead of shaking, required an appreciably longer time before oxidizing the glucose, and correspondingly illustrated rather a resembling aspect in its metabolic pattern with that of the anaerophilic yeast strain No. 39. Thus, the stand culture method might enable this aerophilic yeast to intensify

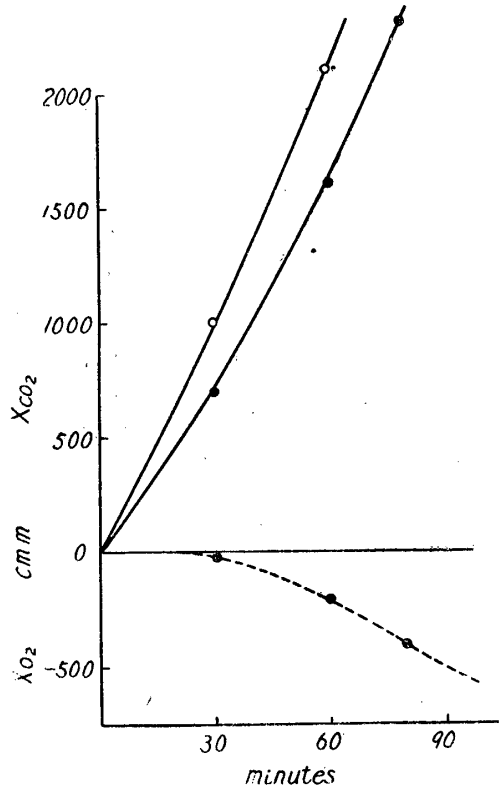


Fig. 1

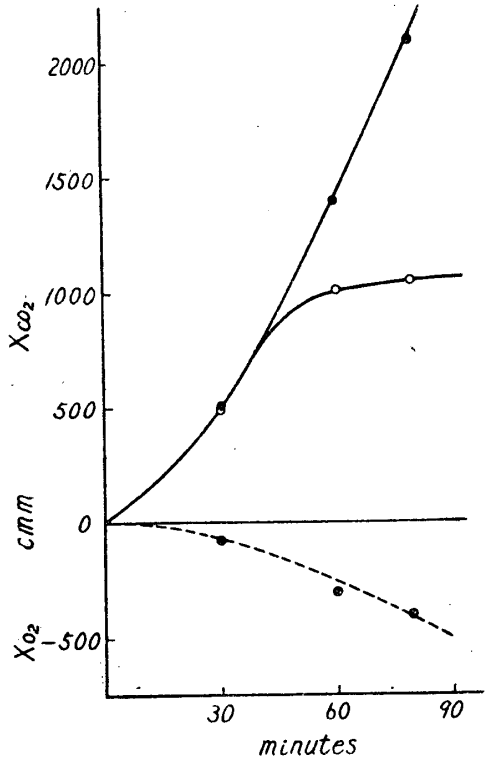


Fig. 2

Fig. 1–2 Output of carbon dioxide in N_2 or air and uptake of oxygen against glucose by the "silage-yeat" in the surface (Fig. 1) and center portions (Fig. 2) in the Silage B of the 4th day : in air (• — •), in N_2 (o — o) and uptake of oxygen. (• --- •) ; at 37°, pH 3.5 (phosphate) ; and molarity of the substrate is M/35. Organisms tested have been harvested from the silage by differential centrifugation.

its anaerophilic activity and alternatively to reduce its oxidative one, but this tendency induced by the culture method may be also inclined by the culture medium employed, since, if this aerophilic yeast was cultured in the glucose-peptone medium with or without yeast extracts even with standing, it showed a shortened time of lag likewise with its shake cultured cells as above cited, though its anaerophilic fermentability was considerably intensified (Figs 3–6). On the other hand, the anaerophilic yeast strain when cultured in the glucose-silage juice regardless of whether with standing or shaking always remained in an inert state for a considerably long period before oxidation against glucose, as shown in Figs. 7 and 8. Considering these metabolic patterns obtained by both aerophilic and anaerophilic laboratory yeasts, it is not decided whether the aerophilic or anaerophilic yeasts occupied prediominant place at the surface portion of the silage B in this phase, since the silage yeast of the surface portion indicated lag for a considerably longer period before its oxidation of glucose, as above described. The metabolic pattern of the inner silage yeast, however,

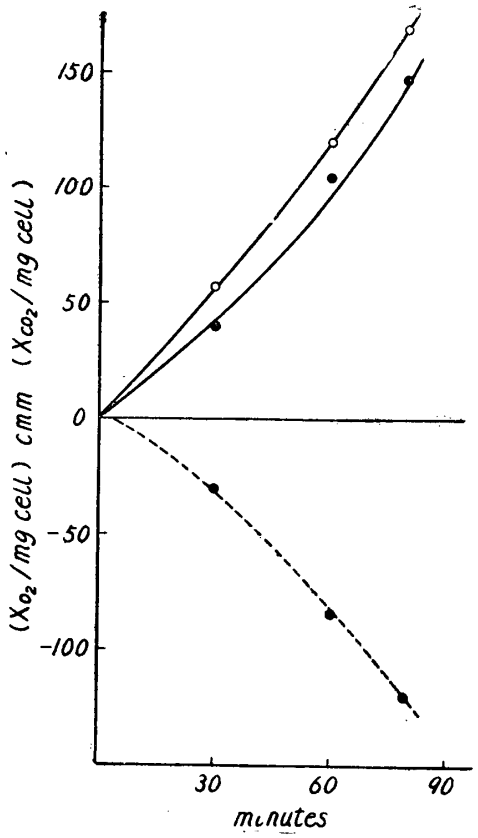
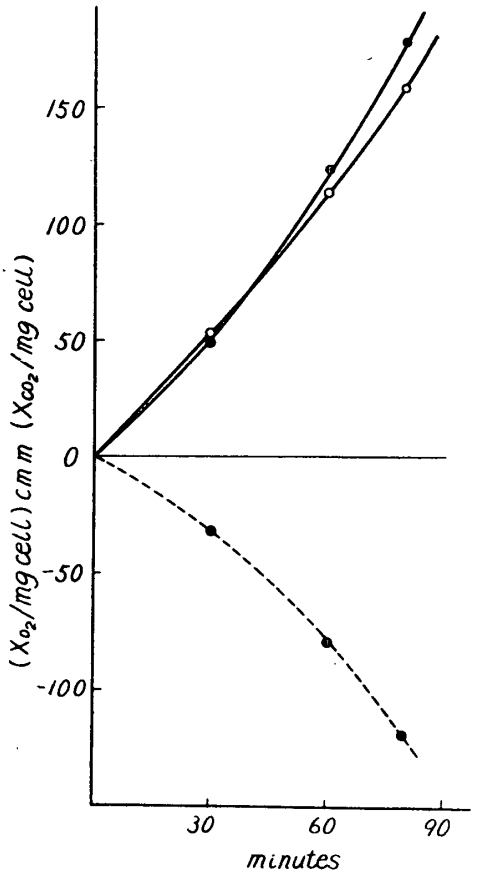


Fig. 3 Output of carbon dioxide in N_2 or air and uptake of oxygen against glucose by the aerophilic yeast strain No. 32; in air (• — •) in N_2 (o — o) and uptake of oxygen (• --- •) ; at 37°, pH 3.5 (phosphate) ; and molarity of the substrate is M/35. Organisms tested have been cultured at 30° for 40 hours in glucose-peptone-yeast extracts medium (1.0 % glucose, 0.5 % bouillon, 0.5 % peptone, 0.1 % yeast extracts and 0.25 % NaCl) with standing.



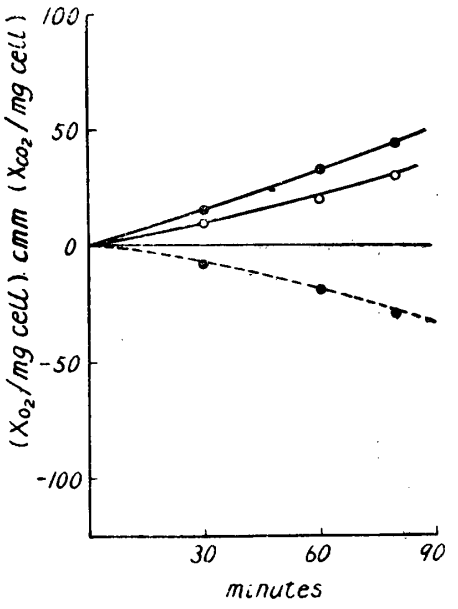


Fig. 4–5 Output of carbon dixoide in N_2 or air and uptake of oxygen against glucose by the aerophilic yeast strain No. 32 ; in air (• — •), in N_2 (o — o) and uptake of oxygen (• --- •) ; at 37°, pH 3.5 (phosphate) ; and molarity of the substrate is M/35. Organisms tested have been cultured at 30° for 40 hours (Fig. 4) or 65 hours (Fig. 5) in glucose-peptone medium (1.0% glucose, 0.5% bouillon, 0.5% peptone and 0.25% NaCl) with standing.

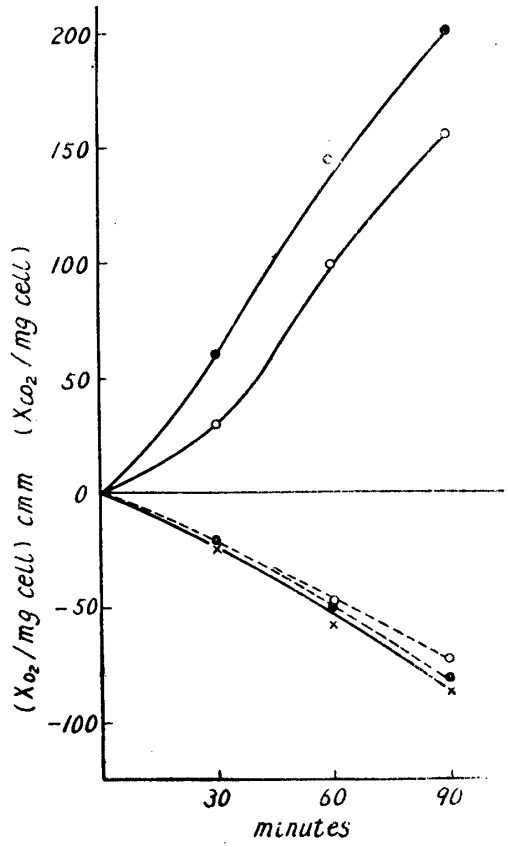


Fig. 6 Output of carbon dioxide agaist glucose in N_2 or air and uptake of oxygen against glucose, lactic and acetic acids by the aerophilic yeast strain No. 32 ; output of carbon dioxide in air (• — •), and in N_2 (o — o), and uptake of oxygen agaist glucose (• --- •), lactic acid (× — ×) and acetic acid (o --- o) ; at 37°, pH 3.5 (phosphate) ; and molarity of the substrates is M/35. Organisms tested have been cultured at 30° for 37 hours in glucose-peptone medium (1.0% glucose, 0.5% bouillon, 0.5% peptone and 0.25% NaCl) with shaking.

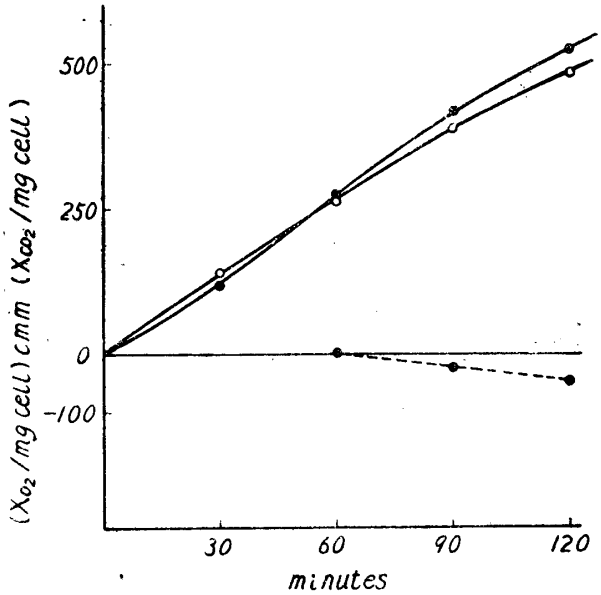


Fig. 7 (Cells were cultured for 64hr. with standing)

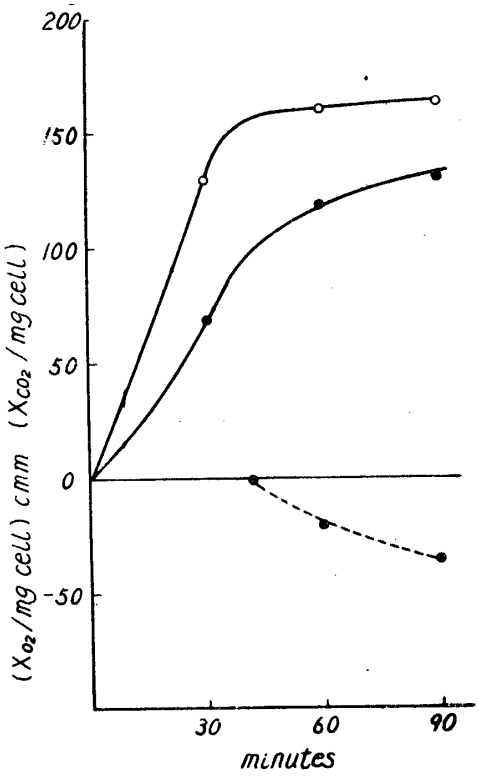


Fig. 8 (cells were cultured for 37hr. with shaking.)

Fig. 7–8 Output of carbon dioxide in N_2 or air and uptake of oxygen angainst glucose by the anaerophilic yeast strain No. 39 ; in air (• — •), in N_2 (o — o) and uptake of oxygen (• --- •) ; at 37°, pH 3.5 (phosphate) ; and molarity of the substrate is M/35. Organisms tested have been cultured at 30° in glucose-silage juice medium with standing (Fig. 7) or shaking (Fig. 8).

rather resembled that of Sake-yeast previously obtained (see Fig 2). It may be then presumed that both in the surface and center portions of this silage B, there would occur exceedingly a growth of an anaerophile-type yeast like the strain No. 39, which may overcome that of an aerophile-type yeast like the strain No. 32. This may be probable because in the initial stage of the ensiling process the heavy weights laid upon the packs in practice might be still effective to keep the anaerobic circumstances even in the surface portion of the silage and also in its center portion.

At any rate, in this phase the most metabolically active yeast throughout the ensiling process was present in both surface and center portions of the silage, and could oxidize free lactic acid or its salt as well as glucose even in an acidic environment (pH 3.5).

It is then reasonable that, if a good aerobic circumstance is established, there occurs a violent digestion of the lactic acid accumulated in the silage by the yeast in its every portion, and thus the yeast may play an inducing rôle to the silage putrefaction.

Phase 2. In this phase there occurred a sudden change in the acid quantity in every portion of the silage and a violent growth of the microbes especially present in its surface portion. The surface yeast also showed a bursting increase in its number, whereas the inner yeast either grew slightly (silage A) or kept almost the same level in its number as that in the preceeding phase (silage B). Then in the surface portion of the silage environmental conditions such as pH, aerobic circumstance, etc., may be supposed to be more favorable for the yeast growth in contrast to those in the inner portion, in which in turn occurred a sudden drop of pH due to growth of the acid forming bacteria, and thus the pH value attained the minimum (pH 3.4~3.5), maintaining this level in a further later stage of the process till the end. On the contrary, pH in the surface portion was either almost unchanged or slightly ascended in this phase. Since the sugar had been then almost exhaustively consumed, the acid there accumulated may be responsible for the bursting growth of the surface yeast. Although an actual decrease of the acid accumulated in the surface portion was observed in this phase, it is doubtful whether the accumulated acid was a good nourishment enabling the surface yeast to grow so violently. However, this accumulated acid may certainly have played a rôle because the respiratory activity of the surface yeast against either free lactic acid or its salt, if in total, was still maintained to be relatively high in this phase, though a marked decrease could be noticed in each cell.

Both surface and inner yeasts in this phase showed a striking drop in their fermentability, as compared with their oxidative activity. Especially, the anaerobic fermentability of the surface yeast was decreased so greatly that it became almost nullified in this phase. The metabolic pattern of this surface

yeast was in a good agreement with that of the laboratory aerophilic yeast when cultured in the glucose-silage juice with shaking, and Xo_2 as well as $X^{air}_{O_2}$ of the former surface yeast showed a great advance over its $X^{N_2}_{CO_2}$ (see Fig. 9–11).

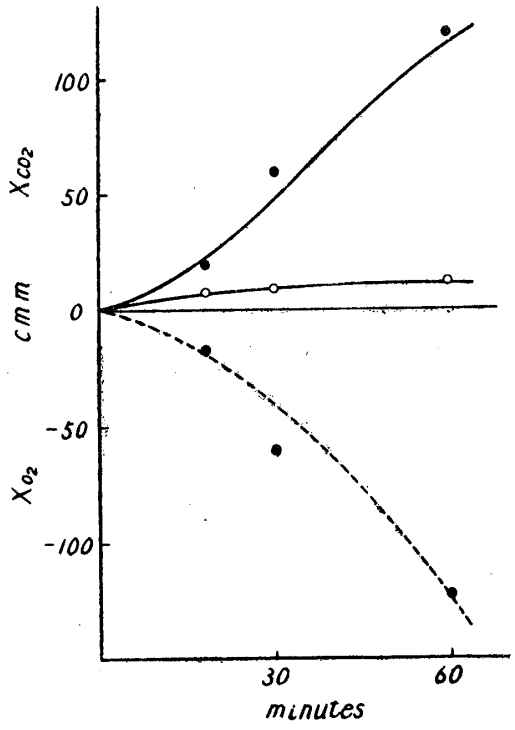


Fig. 9

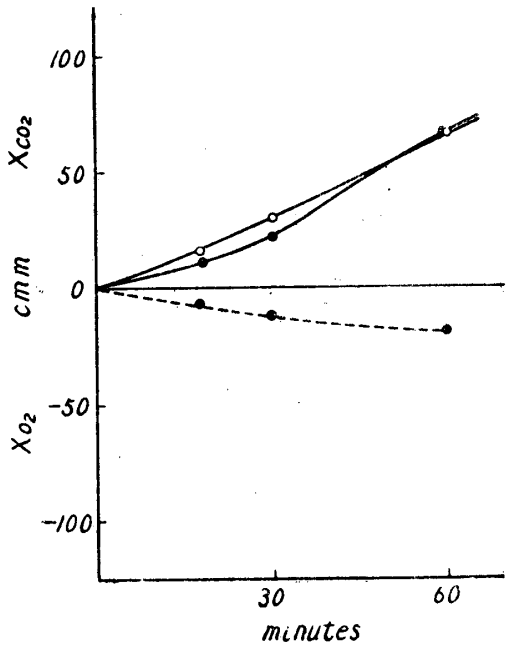


Fig. 10

Fig. 9–10 Output of carbon dioxide in N_2 or air and uptake of oxygen against glucose by the "Silage-yeast" in the surface (Fig. 9) and center portions (Fig. 10) in the Silage B of the 8th day ; in air (• — •), in N_2 (o — o) and uptake of oxygen (• --- •) ; at 37°, pH 3.5 (phosphate) ; and molarity of the substrate is M/35. Organisms tested have been harvested from the silage by differential centrifugation.

Thus, it is obvious that the surface yeast in this phase belonged predominantly to an aerophilic group like a film forming yeast strain, No. 32. This case may successfully demonstrate that from a comparison between metabolic activities of the cells ecologically harvested and those of the laboratory cells of the same origin, a notable knowledge is abtained on the kind of the microbes playing a leading rôle in the actual field.

The anaerobic fermantability of the inner yeast was yet considerably higher than its oxidative activity, and its metabolic pattern in the preceding phase was also well re-established in this phase, though in a limited range. The anaerophilic yeast also kept its dominant place in the center portion of the silage in this phase 2.

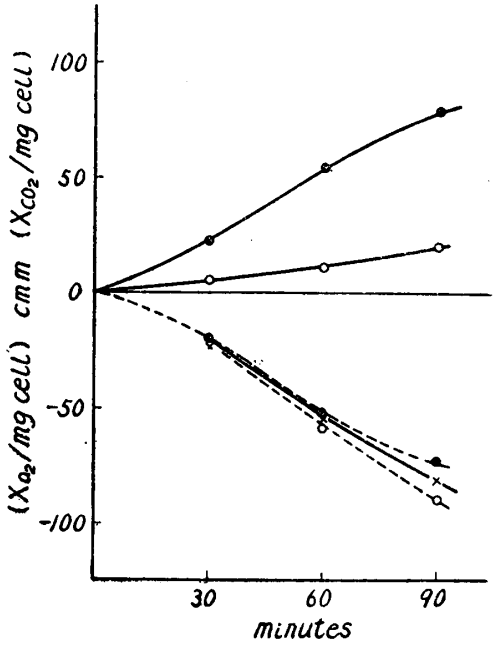


Fig. 11 Output of carbon dioxide against glucose in N_2 or air and uptake of oxygen against glucose, lactic acid and acetic acid by the anerophilic yeast strain No. 39 ; output of carbon dioxide in air (• — •) and in N_2 (o — o), and uptake of oxygen against glucose (• --- •), lactic acid (× — ×) and acetic acid (o — o) ; at 37°, pH 3.5 (phosphate) ; and molarity of the substrates is M/35. Organisms tested have been cultured at 30° for 61 hours in glucose-silage juice medium with shaking.

Phase 3. Microbial actions became faint and therefore changes in the chemical constituents stopped almost perfectly in this phase, though there was a slight increase in the acid quantity at its early stage. The yeast showed also a rapid (in the surface portion) or slight (in the center portion) decrease in its number according to its living place, where the exceeding amount of its number in the surface portion of the silage disappeared and then both surface and inner yeasts became almost equalized in each number in this phase. Faint metabolic activities of the surface yeast were also observed in this phase and therefore no yeast action seemed to occur in this surface portion. Upon investigation of its anaerobic fermentability, a lag period of 60 minutes was then recognized before the output of CO_2 from the added glucose. If this lag period indicates the time required for an adaptation of the yeast to an anaerobic condition, it may be considered to be the time for re-activation of carboxylase of the aerobically cultured anaerophilic yeast under anaerobic conditions, since Terui *et al.* (4) have observed such an anaerobic re-activation of an aerophilic Hansenula yeast. However, the surface portion of the silage in this phase was considerably governed by the aerobic condition, so that there would not occur for such a long time adaptation of the yeast fermentability to the anaerobic condition and therefore fermentation by the yeast there living may not be encountered in this surface portion. Anaerobic fermentability of the inner yeast in this phase was considerably maintained and almost unchanged in contrast to a great decrease in its oxidative activity. However, its aerobic fermentalility in this phase was overcome by its anaerobic one, though still relatively higher than its oxidative activity. This metabolic pattern of the inner yeast agrees

very well with that obtained by the ripe cells of the laboratory anaerophilic yeast, No. 39, as illustrated in Fig. 11. Besides, the Shôyu-Moromi yeast (soy sauce-mash yeast) obtained ecologically in like manner with the silage yeast showed also a prevalence of its anaerobic fermentability over its aerobic one and furthermore, such a prevalence was also observed in ripe cells of an anaerophilic (and osmophilic) yeast strain, *Zygosaccharomyces major* (a typical Shôyu-yeast strain). Meyerhof (5) has once pointed out that after fermentation of sugars by an anaerophilic yeast, *Saccharomyces cerevisiae*, its aerobic fermentability decreases considerably, whilst its oxidative activity increases, but its anaerobic one is almost unchangeable. Thus even an anaerophilic yeast when ripened indicates such a prevalance of its aerophilic character. The anerophilic cells of the inner yeast were then ripened and increased its alternative aerophilic character, despite showing of an unchanged anaerobic fermentalility, in this phase 3.

Phase 4. This phase is a ripening stage of the ensiling process and if an ensiling procedure is kept under good management, there may not occur if not at all, any apperciable changes in the chemical constituents during this stage. However, as seen in the silage A, the pH value of its surface portion ascended so markedly, reaching to 7.4 or more, therfore some putrefactive bacteria may be supposed to play the most important rôle in this portion, in which on the contrary the yeast showed a remarkable decrease in its number and also eliminated metabolic activities. Then it is probably mentioned that the active rôle displayed by the yeast in this surface portion was completely replaced by the putrefactive bacteria in this phase. In the silage B, which was in better ensiling management than the silage A, the ascendance of pH value in its surface portion was not so marked as in that of the silage A, and the yeast from the sample of the 35th day after packing again gained a relative high oxidative activity, though still almost indiscernible fermentability. This surface yeast, showed an oxidative activity against free acetic acid, a phenomenon not noticed in all other surface yeasts treated except that in the phase 2 of the silage A. Eaton and Klein (6) have recently researched on the changes in the acetate oxidation ability of *Sacch. cerevisiae* due to its growth period and found that this ability is recognized only in its younger cell and becomes lost when old. This valuable investgation may certainly serve to account for the above result obtained by the author. At any rate, the fact that the surface yeast can oxidize acetic acid as well as the lactic one though this activity is changeable due to the ensiling stage demonstrates apparently that this yeast participates in the putrefaction of the surface portion of the silage as an inducer to the purtefactive bacteria.

The inner yeast of the silage after passing through the phase 3 shows a great decrease in its number and despite a large loss of its fermentability it

retains considerably its oxidative activity till the end. Thus the yeast of the 35th day after packing showed a relatively intensified attack against the lactic and acetic acids. This oxidative activity which once disappeared became reactivated in the yeast of the 123th day (the last sample). Accordingly, the oxidative activity of the inner yeast was fluctuating within a limited range throughout this phase and thus this yeast, if placed under aerobic conditions, might still be able to digest oxidatively the acid and to supply a clue to the putrefaction of the silage. On the other hand, this inner yeast at the early stage of this ripening phase showed a lag period before the fermentation and finally lost its fermentability alomst entirely when nearing the end. It is noteworthy to mention that in both surface and center yeasts of the silage their fermentability has been ruined at the relatively earlier stage of the ripening phase, whilst their oxidative activity was considerably maintained till the last.

Discussion

When any microbial action in the actual field is to be researched, the organisms are usually isolated by using some appropriate culture medium, which in turn may significantly affect the physiological properties of the actual microbes fact. To remove such a difficulty in the culture method, a differential centrifugation technique has been applied in this laboratory for isolation of the organisms and by this means various actions of the yeast present in Sake- and Shôyu-Moromi have been precisely investigated. This technique was also employed and several abilities of the yeast, the population of which periodically showed a large fluctuation in the ensiling process, were observed in this research. As a result, the silage-yeast, either of the surface or inner yeasts, possessed an oxidative ability against the lactic acid as well as glucose throughout the process, whilst its fermentability became almost nullified in the later period of this process. Besides, the acetic acid oxidation is either not at all or only slightly seen in those silage-yeast cells periodically harvested. This may account for the fact that the quantity of the acetic acid accumulated was rarely changeable throughout the process, though a fluctuation of quantity of the lactic acid accumulated was occassionally observed. When cells of the laboratory yeast strain No. 39 were harvested from the stand culture in the pressed juice of the silage with glucose, these cells showed no oxidation against the acetic acid, whereas, in contrast, there was seen a considerable oxidation against the glucose. When these cells were harvested from the shake culture of 37 hours incubation, they exerted appreciable oxidation against both acetic acid and gulcose. The aged silage would contain a certain factor which may prevent achievement of the oxidative activity against the acetic acid by the yeast cells as cultured with standing. This is expected to be confirmed by further investigation in the future. The results obtained also verified the previously stated assumption, that the yeast may display

a significant rôle in the putrefactive process of the silage, and correspondingly conversion of the fermentation into the respiration in the yeast cells under aerobic condition. This conversion mechanism has been recently researched by several investigators and it was occassionally pointed out that the an aerobically grown yeast cells lose the ability to oxidize glucose because of their lack of respiratory enzyme, and oxygen induces their resynthesis with resulting restoration of the capacity for glucose oxidation (7). Since whether the oxidation substrate is glucose added or ethanol formed was not determined in the test of this research, it is still doubtful that the shift of anaerobiosis to aeroboisis in the silage yeast tested under aerobic conditions takes place simply due to such an induction of oxygen. The concept of this kind, however, may provide some evidence to clarify the puterfying mechanism of the silage.

On the other hand, when any ability of the yeast cells ecologically harvested is allowed to be compared with that of the laboratory yeast cells of the same origin, it is then recognized by the author that this comparison technique may serve to determine the kind of yeast that is playing the major rôle in the process of the concerned microbial fact. That the plate method by using a defined medium, based upon the nutritional requirements of the microbes therein present, is also useful for this purpose, has been once encountered by Lochhead *et al.* in their research regarding soil microbes. Each of these techniques, if in combination, may become a more powerful means in elucidation of the concerned ecological problem.

Summary

From two kinds of the silage prepared with green maize and soybean, the yeast was directly separated by differential centrifugation without using the culture medium and then subject to investigation of its oxidative or aerobic as well as anaerobic fermentative abilities. The ensiling process was divided into four phases, based upon the metabolic activities of the yeast present in each silage sample as well as the changes in its constituents. Such an ecological result on the metabolic activites of the socalled silage-yeast was refered to that obtained by laboratory yeasts previously isolated purely from the silage by the using culture medium.

At the initial stage of the ensiling process, from its begining to the 4th day after packing, the most metabolically active yeast throughout the process was obtained in both its surface and center portions, and could oxidize free lactic acid as well as glucose even in an acidic environment such as pH 3.5.

In the second phase, the surface yeast (yeast present in the surface portion of the silage) showed a remarkable increase in its number, while the inner yeast (yeast prsent in the center portion of the silage) grew slightly, as compared with the yeast cell number of both portions in the preceeding phase 1. The surface

yeast in this phase belongs to an aerophilic one, based upon the comparison with the metabolic pattern of the laboratory aerophilic yeast. On the other hand, since the aerobic fermentability of the inner yeast was yet higher than its respiratory acitvity, an anaerophilic yeast dominantly occupied the inner portion of the silage in the phase 2. Successively to the previous stage, the metabolic activities of the silage yeast was continued to become faint. However, the anaerobic fermentability of the inner yeast was almost unchanged in contrast to decrease in its oxidative one in the phase 3.

The oxidative activity of both surface and inner yeasts was considerably remained till the end, whereas in contrast their fermentability was almost lost at the earlier stage of the last phase.

The previously stated assumption that the yeast may display a significant rôle in the putrefaction of the silage was confirmed from the results above obtained.

The author wishes to thank Professor T. Uemura for suggesting this line of work and for his helpful advice. Thanks are also due to the members of the Kawatabi Farm of the Tohoku University for their support during this investigation.

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